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Flk-1 IS A RECEPTOR FOR VASCULAR
ENDOTHELIAL GROWTH FACTOR

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Flk-1 IS A RECEPTOR FOR VASCULAR
ENDOTHELIAL GROWTH FACTOR

1. INTRODUCTION

5 The present invention relates to the use of ligands
for the FLK-1 receptor for the modulation of angiogenesis
and vasculogenesis. The invention is based, in part, on
the demonstration that Flk-1 tyrosine kinase receptor
expression is associated with endothelial cells and the
10 identification of vascular endothelial growth factor
(VEGF) as the high affinity ligand of Flk-1. These
results indicate a major role for Flk-1 in the signaling
system during vasculogenesis and angiogenesis.
Engineering of host cells that express Flk-1 and the uses
15 of expressed Flk-1 to evaluate and screen for drugs and
analogs of VEGF involved in Flk-1 modulation by either
agonist or antagonist activities is described.

20 The invention also relates to the use of FLK-1
ligands, including VEGF agonists and antagonists, in the
treatment of disorders, including cancer, by modulating
vasculogenesis and angiogenesis.

2. BACKGROUND OF THE INVENTION

25 Receptor tyrosine kinases comprise a large family of
transmembrane receptors for polypeptide growth factors
with diverse biological activities. Their intrinsic
tyrosine kinase function is activated upon ligand
binding, which results in phosphorylation of the receptor
and multiple cellular substrates, and subsequently in a
30 variety of cellular responses (Ullrich A. and
Schlessinger, J., 1990, Cell 61:203-212).

35 A receptor tyrosine kinase cDNA, designated fetal
liver kinase 1 (Flk-1), was cloned from mouse cell
populations enriched for hematopoietic stem and
progenitor cells. The receptor was suggested to be
involved in hematopoietic stem cell renewal (Matthews

et al., 1991, Proc. Natl. Acad. Sci. USA 88:9026-9030). Sequence analysis of the Flk-1 clone revealed considerable homology with the c-Kit subfamily of receptor kinases and in particular to the *Flt* gene product. These receptors all have in common an extracellular domain containing immunoglobulin-like structures.

The formation and spreading of blood vessels, or vasculogenesis and angiogenesis, respectively, play important roles in a variety of physiological processes such as embryonic development, wound healing, organ regeneration and female reproductive processes such as follicle development in the corpus luteum during ovulation and placental growth after pregnancy. Uncontrolled angiogenesis can be pathological such as in the growth of solid tumors that rely on vascularization for growth.

Angiogenesis involves the proliferation, migration and infiltration of vascular endothelial cells, and is likely to be regulated by polypeptide growth factors. Several polypeptides with in vitro endothelial cell growth promoting activity have been identified. Examples include acidic and basic fibroblastic growth factor, vascular endothelial growth factor and placental growth factor. Although four distinct receptors for the different members of the FGF family have been characterized, none of these have as yet been reported to be expressed in blood vessels in vivo.

While the FGFs appear to be mitogens for a large number of different cell types, VEGF has recently been reported to be an endothelial cell specific mitogen (Ferrara, N. and Henzel, W.J., 1989, Biochem. Biophys. Res. Comm. 161:851-858). Recently, the *fms*-like tyrosine receptor, *flt*, was shown to have affinity for VEGF (DeVries, C. et al., 1992, Science 255:989-991).

3. SUMMARY OF THE INVENTION

The present invention relates to the use of ligands for the FLK-1 receptor for the modulation of angiogenesis and vasculogenesis. The present invention is based, in part, on the discovery that the Flk-1 tyrosine kinase receptor is expressed on the surface of endothelial cells and the identification of vascular endothelial growth factor (VEGF) as the high affinity ligand of Flk-1. The role of endothelial cell proliferation and migration during angiogenesis and vasculogenesis indicate an important role for Flk-1 in these processes. The invention is described by way of example for the murine Flk-1, however, the principles may be applied to other species including humans.

Pharmaceutical reagents designed to inhibit the Flk-1/VEGF interaction may be useful in inhibition of tumor growth. VEGF and/or VEGF agonists may be used to promote wound healing. The invention relates to expression systems designed to produce Flk-1 protein and/or cell lines which express the Flk-1 receptor. Expression of soluble recombinant Flk-1 protein may be used to screen peptide libraries for molecules that inhibit the Flk-1/VEGF interaction. Engineered cell lines expressing Flk-1 on their surface may be advantageously used to screening and identifying VEGF agonists and antagonists.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Comparison of the Flk-1 amino acid sequence with related RTKs. Amino acid sequence comparison of Flk-1 with human KDR and rat TKr-C. A section of the sequence which is known for all three receptors is compared and only differences to the Flk-1 sequence are shown.

FIG. 2. Northern blot analysis of Flk-1 gene expression. (A) Expression of Flk-1 RNA in day 9.5 to day 18.5 mouse embryos. Samples (10 μ g) of total RNA from whole mouse embryos were analyzed in each lane.

5 Positions of 28S and 18S ribosomal RNAs are marked.

(B) Expression of Flk-1 mRNA in postnatal day 4 and adult brain in comparison with capillary fragments from postnatal day 4 brain. 1 μ g of poly (A⁺) RNA was loaded on each lane. The 5' 2619 bp of the Flk-1 cDNA were used
10 as a probe. Control hybridization with a GAPDH cDNA probe is shown in the lower panel.

FIG. 3. Abundant Flk-1 gene expression in embryonic tissues. *In situ* hybridization analysis of Flk-1 expression in day 14.5 mouse embryo. (A) Bright
15 field illumination of a parasagittal section through the whole embryo hybridized with a ³⁵S-labeled antisense probe (5' 2619 bp). (B) Dark field illumination of the same section. (C) Control hybridization of an adjacent section with a sense probe. Abbreviations: Ao, aorta;
20 At, atrium; L, lung; Li, liver; Ma, mandible; Mn, meninges; Ms, mesencephalon; T, telencephalon; V, ventricle; Vt, vertebrae.

FIG. 4. Expression of Flk-1 RNA in embryonic organs is restricted to specific cells. Expression of
25 Flk-1 RNA in a day 14.5 mouse embryo at higher magnification. (A) The heart region was probed with a ³⁵S-labeled antisense probe. (B) Adjacent section hybridized with the sense probe. (C) Part of the aorta wall shown on the cellular level. The endothelial cell-
30 layer is indicated by an arrow. (D) The lung, probed with the Flk-1 antisense probe. (E) Control hybridization of an adjacent section hybridized with the sense probe. Abbreviations: At, atrium; B, bronchus; Ed, endothelial cell layer; En, endocardium; L, lung, Li,

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liver; Lu, lumina of the aorta; Ml, muscular; My, myocardium.

FIG. 5. Flk-1 gene expression in the brain of the developing mouse. *In situ* hybridization analysis of Flk-1 gene expression in the brain at different developmental stages. All sections were probed with the Flk-1 antisense probe. (A) Sagittal section of the telencephalon of a day 11.5 mouse embryo. A single blood vessel expressing Flk-1, which sprouts from the meninges into the neuroectoderm, is indicated by an arrow. (B) Sagittal sections of the brain of embryo day 14.5 and (C) of postnatal day 4. Shown are regions of the mesencephalon. Branching capillaries and blood vessels expressing Flk-1 are indicated by an arrow. (D) Sagittal section of an adult brain; a region of the mesencephalon is shown. Cells expressing Flk-1 are indicated by an arrow. Abbreviations: M, meninges; V, ventricle;

FIG. 6. Expression of Flk-1 in the choroid plexus of adult brain. (A) Darkfield illumination of the choroid plexus of an adult mouse brain hybridized with Flk-1 antisense probe. (B) Choroid plexus shown at a higher magnification. Arrows indicate single cells, which show strong expression of Flk-1. Abbreviations: CP, choroid plexus; E, ependyme; Ep, epithelial cells; V, ventricle.

FIG. 7. Flk-1 is expressed in the glomeruli of the kidney. (A) Parasagittal section of a 4-day postnatal kidney, hybridized with the Flk-1 antisense probe. Hybridization signal accumulates in the glomeruli, as indicated by arrowheads. (B) Control hybridization of an adjacent section with the sense probe. (C) Sagittal section of an adult kidney probed with Flk-1. Arrowheads indicate glomeruli. (D) Glomerulus of an adult kidney at a higher magnification. The arrows in (A) and (D)

indicate cells aligned in strands in the juxtaglomerular region expressing Flk-1.

FIG. 8. *In situ* hybridization analysis of Flk-1 expression in early embryos and extraembryonic tissues.

- 5 (A) Sagittal section of a day 8.5 mouse embryo in the maternal deciduum probed with Flk-1. (B) Higher magnification of the deciduum. Arrowheads indicate the endothelium of maternal blood vessels strongly expressing Flk-1 RNA. (C) High magnification of the yolk sac and
10 the trophoctoderm of a day 9.5 mouse embryo. (D) High magnification of a blood island. Abbreviations:
A, allantois; Bi, blood island; Bv, maternal blood vessel; D, deciduum; En, endodermal layer of yolk sac; M, mesenchyme; Ms, mesodermal layer of yolk sac; NF,
15 neural fold; T, trophoblast; Y, yolk sac.

- FIG. 9. Flk-1 is a receptor for VEGF. (A) Cross linking of ^{125}I -VEGF to COS cells transiently expressing the Flk-1 receptor and control cells were incubated with ^{125}I -VEGF at 4°C overnight, then washed twice with
20 phosphate buffered saline (PBS) and exposed to 0.5 mM of the cross linking agent DSS in PBS for 1 hour at 4°C. The cells were lysed, Flk-1 receptor immunoprecipitated, and analyzed by polyacrylamide gel electrophoresis followed by autoradiography. Molecular size markers are
25 indicated in kilodaltons. (B) Specific binding of ^{125}I -VEGF to COS cells expressing Flk-1. COS cells transiently expressing Flk-1 were removed from the plate and resuspended in binding medium (DMEM, 25 mM Hepes, 0.15% gelatin). Binding was performed at 15°C for 90
30 minutes in a total volume of 0.5 ml containing 2×10^5 cells, 15,000 cpm ^{125}I -VEGF, and the indicated concentrations of unlabeled ligand. The cells were washed twice with PBS / 0.1% BSA and counted in a gamma
35 counter.

FIG. 10. VEGF-induced autophosphorylation of Flk-1. COS cells transiently expressing Flk-1 receptor and control cells were starved for 24 hours in DMEM containing 0.5% fetal calf serum and then stimulated with VEGF for 10 minutes as indicated. The cells were solubilized, Flk-1 receptor immunoprecipitated with a polyclonal antibody against its C-terminus, separated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The blot was probed with antiphosphotyrosine antibodies (5B2). The protein bands were visualized by using a horseradish-peroxidase coupled secondary antibody and BCL™ (Amersham) detection assay.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of ligands for the FLK-1 receptor to modulate angiogenesis and/or vasculogenesis. The invention also involves the expression of Flk-1 to evaluate and screen for drugs and analogs of VEGF that may be involved in receptor activation, regulation and uncoupling. Such regulators of Flk-1 may be used therapeutically. For example, agonists of VEGF may be used in processes such as wound healing; in contrast, antagonists of VEGF may be used in the treatment of tumors that rely on vascularization for growth.

The invention, is based, in part, on results from *in situ*-hybridization and Northern blot analyses indicating that Flk-1 is an endothelial cell specific RTK. In addition, cross-linking experiments have shown Flk-1 to be a high affinity receptor for vascular endothelial growth factor (VEGF), indicating that Flk-1 plays a crucial role in the development and differentiation of hemangioblast and in subsequent endothelial cell growth during vasculogenesis and angiogenesis.

As explained in the working examples, infra, the polymerase chain reaction (PCR) method was used to isolate new receptor tyrosine kinases specifically expressed in post-implantation embryos and endothelial
5 cells. One such clone was found to encode a RTK that had previously been isolated from populations of cells enriched for hematopoietic cells and designated fetal liver kinase-1 (Flk-1) (Matthews et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88:9026-9030).

10 For clarity of discussion, the invention is described in the subsections below by way of example for the murine Flk-1. However, the principles may be analogously applied to clone and express the Flk-1 of other species including humans.

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5.1. THE Flk-1 CODING SEQUENCE

The nucleotide coding sequence and deduced amino acid sequence of the murine Flk-1 gene has recently been described (Matthews et al., 1991, Proc. Natl. Acad. Sci.
20 U.S.A., 88:9026-9030). In accordance with the invention, the nucleotide sequence of the Flk-1 protein or its functional equivalent in mammals, including humans, can be used to generate recombinant molecules which direct the expression of Flk-1; hereinafter, this receptor will
25 be referred to as "Flk-1", regardless of the species from which it is derived.

In a specific embodiment described herein, the murine Flk-1 gene was isolated by performing a polymerase chain reaction (PCR) using two degenerate oligonucleotide
30 primer pools that were designed on the basis of highly conserved sequences within the kinase domain of receptor tyrosine kinases (Hanks et al., 1988,) As a template, DNA from a λ gt10 cDNA library prepared from day 8.5 mouse embryos, was used. In a parallel approach, similar
35 primers were used to amplify RTK cDNA sequences from

capillary endothelial cells that had been isolated from the brains of post-natal day 4-8 mice. This is a time when brain endothelial cell proliferation is maximal.

Both approaches yielded cDNA sequences encoding the

5 recently described fetal liver RTK, Flk-1 (Matthews et al., 1991). Based on amino acid homology, this receptor is a member of the type III subclass of RTKs (Ullrich and Schlessinger) which contain immunoglobulin-like repeats in their extracellular domains (FIG. 1).

10 The invention also relates to Flk-1 genes isolated from other species, including humans, in which Flk-1 activity exists. Members of the Flk-1 family are defined herein as those receptors that bind VEGF or fragments of the peptide. Such receptors may demonstrate about 80%
15 homology at the amino acid level in substantial stretches of DNA sequence. A bacteriophage cDNA library may be screened, under conditions of reduced stringency, using a radioactively labeled fragment of the mouse Flk-1 clone. Alternatively the mouse Flk-1 sequence can be used to
20 design degenerate or fully degenerate oligonucleotide probes which can be used as PCR probes or to screen bacteriophage cDNA libraries. A polymerase chain reaction (PCR) based strategy may be used to clone human Flk-1. Two pools of degenerate oligonucleotides,
25 corresponding to a conserved motifs between the mouse Flk-1 and receptor tyrosine kinases, may be designed to serve as primers in a PCR reaction. The template for the reaction is cDNA obtained by reverse transcription of mRNA prepared from cell lines or tissue known to express
30 human Flk-1. The PCR product may be subcloned and sequenced to insure that the amplified sequences represent the Flk-1 sequences. The PCR fragment may be used to isolate a full length Flk-1 cDNA clone by radioactively labeling the amplified fragment and
35 screening a bacteriophage cDNA library. Alternatively,

the labeled fragment may be used to screen a genomic library. For a review of cloning strategies which may be used, see e.g., Maniatis, 1989, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press, N.Y.; and
5 Ausubel et al., 1989, Current Protocols in Molecular Biology, (Green Publishing Associates and Wiley Interscience, N.Y.)

Isolation of a human Flk-1 cDNA may also be achieved by construction of a cDNA library in a mammalian
10 expression vector such as pcDNA1, that contains SV40 origin of replication sequences which permit high copy number expression of plasmids when transferred into COS cells. The expression of Flk-1 on the surface of
15 transfecting COS cells may be detected in a number of ways, including the use of a labeled ligand such as VEGF or a VEGF agonist labeled with a radiolabel, fluorescent label or an enzyme. Cells expressing the human Flk-1 may be enriched by subjecting transfecting cells to a FACS (fluorescent activated cell sorter) sort.

20 In accordance with the invention, Flk-1 nucleotide sequences which encode Flk-1, peptide fragments of Flk-1, Flk-1 fusion proteins or functional equivalents thereof may be used to generate recombinant DNA molecules that direct the expression of Flk-1 protein or a functionally
25 equivalent thereof, in appropriate host cells. Alternatively, nucleotide sequences which hybridize to portions of the Flk-1 sequence may also be used in nucleic acid hybridization assays, Southern and Northern blot analyses, etc.

30 Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used in the practice of the invention for the cloning and expression of the Flk-1 protein. Such DNA sequences

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include those which are capable of hybridizing to the murine Flk-1 sequence under stringent conditions.

Altered DNA sequences which may be used in accordance with the invention include deletions, additions or
5 substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product itself may contain deletions, additions or substitutions of amino acid residues within the Flk-1 sequence, which result in
10 a silent change thus producing a functionally equivalent Flk-1. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipatic nature of the residues involved. For example, negatively
15 charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine; glycine, alanine;
20 asparagine, glutamine; serine, threonine; phenylalanine, tyrosine. As used herein, a functionally equivalent Flk-1 refers to a receptor which binds to VEGF or fragments, but not necessarily with the same binding affinity of its counterpart native Flk-1.

25 The DNA sequences of the invention may be engineered in order to alter the Flk-1 coding sequence for a variety of ends including but not limited to alterations which modify processing and expression of the gene product. For example, mutations may be introduced using techniques
30 which are well known in the art, e.g. site-directed mutagenesis, to insert new restriction sites, to alter glycosylation patterns, phosphorylation, etc. For example, in certain expression systems such as yeast, host cells may over glycosylate the gene product. When
35 using such expression systems it may be preferable to

alter the Flk-1 coding sequence to eliminate any N-linked glycosylation site.

In another embodiment of the invention, the Flk-1 or a modified Flk-1 sequence may be ligated to a
5 heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries it may be useful to encode a chimeric Flk-1 protein expressing a heterologous epitope that is recognized by a commercially available antibody. A fusion protein may also be
10 engineered to contain a cleavage site located between the Flk-1 sequence and the heterologous protein sequence, so that the Flk-1 can be cleaved away from the heterologous moiety.

In an alternate embodiment of the invention, the
15 coding sequence of Flk-1 could be synthesized in whole or in part, using chemical methods well known in the art. See, for example, Caruthers, et al., 1980, Nuc. Acids Res. Symp. Ser. 7:215-233; Crea and Horn, 180, Nuc. Acids Res. 9(10):2331; Matteucci and Caruthers, 1980,
20 Tetrahedron Letters 21:719; and Chow and Kempe, 1981, Nuc. Acids Res. 9(12):2807-2817. Alternatively, the protein itself could be produced using chemical methods to synthesize the Flk-1 amino acid sequence in whole or in part. For example, peptides can be synthesized by
25 solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography. (E.g., see Creighton, 1983, Proteins Structures And Molecular Principles, W.H. Freeman and Co., N.Y. pp. 50-60). The composition of the synthetic
30 peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see Creighton, 1983, Proteins, Structures and Molecular Principles, W.H. Freeman and Co., N.Y., pp. 34-49.

5.2. EXPRESSION OF Flk-1 RECEPTOR AND GENERATION OF CELL LINES THAT EXPRESS Flk-1

In order to express a biologically active Flk-1, the nucleotide sequence coding for Flk-1, or a functional equivalent as described in Section 5.1 supra, is inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. The Flk-1 gene products as well as host cells or cell lines transfected or transformed with recombinant Flk-1 expression vectors can be used for a variety of purposes. These include but are not limited to generating antibodies (i.e., monoclonal or polyclonal) that bind to the receptor, including those that competitively inhibit binding of VEGF and "neutralize" activity of Flk-1 and the screening and selection of VEGF analogs or drugs that act via the Flk-1 receptor; etc.

5.2.1. EXPRESSION SYSTEMS

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the Flk-1 coding sequence and appropriate transcriptional/translational control signals. These methods include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.

A variety of host-expression vector systems may be utilized to express the Flk-1 coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA,

plasmid DNA or cosmid DNA expression vectors containing the Flk-1 coding sequence; yeast transformed with recombinant yeast expression vectors containing the Flk-1 coding sequence; insect cell systems infected with
5 recombinant virus expression vectors (e.g., baculovirus) containing the Flk-1 coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression
10 vectors (e.g., Ti plasmid) containing the Flk-1 coding sequence; or animal cell systems infected with recombinant virus expression vectors (e.g., adenovirus, vaccinia virus) including cell lines engineered to contain multiple copies of the Flk-1 DNA either stably
15 amplified (e.g., CHO/dhfr) or unstably amplified in double-minute chromosomes (e.g., murine cell lines).

The expression elements of these systems vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable
20 transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage λ , plac, ptrp, ptac (ptrp-lac hybrid
25 promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedrin promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (e.g., heat shock promoters; the promoter for the
30 small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of
35 mammalian cells (e.g., metallothionein promoter) or from

mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the Flk-1 DNA SV40-, BPV- and EBV-based vectors may be used
5 with an appropriate selectable marker.

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the Flk-1 expressed. For example, when large quantities of Flk-1 are to be produced for the
10 generation of antibodies or to screen peptide libraries, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the E. coli expression vector pUR278 (Ruther et al.,
15 1983, EMBO J. 2:1791), in which the Flk-1 coding sequence may be ligated into the vector in frame with the lac Z coding region so that a hybrid AS-lac Z protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J.
20 Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to
25 glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety.

30 In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987,
35 Expression and Secretion Vectors for Yeast, in Methods in

Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in

- 5 Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast *Saccharomyces*, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II.

- In cases where plant expression vectors are used,
10 the expression of the Flk-1 coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987,
15 EMBO J. 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, EMBO J. 3:1671-1680; Broglie et al., 1984, Science 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, Mol.
20 Cell. Biol. 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques see, for example, Weissbach & Weissbach,
25 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9.

- An alternative expression system which could be used
30 to express Flk-1 is an insect system. In one such system, Autographa californica nuclear polyhidrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The Flk-1 coding sequence may be cloned into non-essential
35 regions (for example the polyhedrin gene) of the virus

and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the Flk-1 coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed. (E.g., see Smith et al., 1983, J. Virol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the Flk-1 coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing Flk-1 in infected hosts. (E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659). Alternatively, the vaccinia 7.5K promoter may be used. (See, e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett et al., 1984, J. Virol. 49:857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. 79:4927-4931).

Specific initiation signals may also be required for efficient translation of inserted Flk-1 coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire Flk-1 gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the

Flk-1 coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the Flk-1 coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cells lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the Flk-1 may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the Flk-1 DNA controlled by appropriate

expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be
5 allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci
10 which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the Flk-1 on the cell surface, and which respond to VEGF mediated signal transduction. Such engineered cell lines are particularly useful in
15 screening VEGF analogs.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski,
20 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk, hgp^rt or ap^rt cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers
25 resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981), Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to
30 the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hyg^r, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize
35 indole in place of tryptophan; hisD, which allows cells

to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-

- 5 (difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

10 5.2.2. IDENTIFICATION OF TRANSFECTANTS OR
TRANSFORMANTS THAT EXPRESS THE Flk-1

The host cells which contain the coding sequence and which express the biologically active gene product may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence
15 of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of Flk-1 mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity.

20 In the first approach, the presence of the Flk-1 coding sequence inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the Flk-1 coding sequence, respectively, or portions or
25 derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to
30 antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the Flk-1 coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the Flk-1 coding sequence can be
35 identified by the absence of the marker gene function.

Alternatively, a marker gene can be placed in tandem with the Flk-1 sequence under the control of the same or different promoter used to control the expression of the Flk-1 coding sequence. Expression of the marker in
5 response to induction or selection indicates expression of the Flk-1 coding sequence.

In the third approach, transcriptional activity for the Flk-1 coding region can be assessed by hybridization assays. For example, RNA can be isolated and analyzed by
10 Northern blot using a probe homologous to the Flk-1 coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

15 In the fourth approach, the expression of the Flk-1 protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation, enzyme-linked immunoassays and the like. The ultimate test of the success of the
20 expression system, however, involves the detection of the biologically active Flk-1 gene product. A number of assays can be used to detect receptor activity including but not limited to VEGF binding assays; and VEGF biological assays using engineered cell lines as the test
25 substrate.

5.3. USES OF THE Flk-1 RECEPTOR AND ENGINEERED CELL LINES

Angiogenesis, the growth of new blood capillary
30 vessels, is required for a number of physiological processes ranging from wound healing, tissue and organ regeneration, placental formation after pregnancy and embryonic development. Abnormal proliferation of blood vessels is an important component of a variety of
35 diseases such rheumatoid arthritis, retinopathies, and

psoriasis. Angiogenesis is also an important factor in the growth and metastatic activity of solid tumors that rely on vascularization. Therefore, inhibitors of angiogenesis may be used therapeutically for the

- 5 treatment of diseases resulting from or accompanied by abnormal growth of blood vessels and for treatments of malignancies involving growth and spread of solid tumors.

- In an embodiment of the invention the Flk-1 receptor and/or cell lines that express the Flk-1 receptor may be
10 used to screen for antibodies, peptides, or other ligands that act as agonists or antagonists of angiogenesis or vasculogenesis mediated by the Flk-1 receptor. For example, anti-Flk-1 antibodies capable of neutralizing the activity of VEGF, may be used to inhibit Flk-1
15 function. Additionally, anti-Flk-1 antibodies which mimic VEGF activity may be selected for uses in wound healing. Alternatively, screening of peptide libraries with recombinantly expressed soluble Flk-1 protein or cell lines expressing Flk-1 protein may be useful for
20 identification of therapeutic molecules that function by inhibiting the biological activity of Flk-1.

- In an embodiment of the invention, engineered cell lines which express the entire Flk-1 coding region or its ligand binding domain may be utilized to screen and
25 identify VEGF antagonists as well as agonists. Synthetic compounds, natural products, and other sources of potentially biologically active materials can be screened in a number of ways. The ability of a test compound to inhibit binding of VEGF to Flk-1 may be measured using
30 standard receptor binding techniques, such as those described in Section 6.1.9. The ability of agents to prevent or mimic, the effect of VEGF binding on signal transduction responses on Flk-1 expressing cells may be measured. For example, responses such as activation of
35 Flk-1 kinase activity, modulation of second messenger

production or changes in cellular metabolism may be monitored. These assays may be performed using conventional techniques developed for these purposes.

5 5.3.1. SCREENING OF PEPTIDE LIBRARY WITH
 Flk-1 PROTEIN OR ENGINEERED CELL LINES

Random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to
10 bind to the ligand binding site of a given receptor or other functional domains of a receptor such as kinase domains (Lam, K.S. et al., 1991, Nature 354: 82-84). The screening of peptide libraries may have therapeutic value in the discovery of pharmaceutical agents that act to
15 inhibit the biological activity of receptors through their interactions with the given receptor.

Identification of molecules that are able to bind to the Flk-1 may be accomplished by screening a peptide library with recombinant soluble Flk-1 protein. Methods
20 for expression and purification of Flk-1 are described in Section 5.2.1 and may be used to express recombinant full length Flk-1 or fragments of Flk-1 depending on the functional domains of interest. For example, the kinase and extracellular ligand binding domains of Flk-1 may be
25 separately expressed and used to screen peptide libraries.

To identify and isolate the peptide/solid phase support that interacts and forms a complex with Flk-1, it is necessary to label or "tag" the Flk-1 molecule. The
30 Flk-1 protein may be conjugated to enzymes such as alkaline phosphatase or horseradish peroxidase or to other reagents such as fluorescent labels which may include fluorescein isothiocyanate (FITC), phycoerythrin (PE) or rhodamine. Conjugation of any given label, to
35 Flk-1, may be performed using techniques that are routine

in the art. Alternatively, Flk-1 expression vectors may be engineered to express a chimeric Flk-1 protein containing an epitope for which a commercially available antibody exist. The epitope specific antibody may be
5 tagged using methods well known in the art including labeling with enzymes, fluorescent dyes or colored or magnetic beads.

The "tagged" Flk-1 conjugate is incubated with the random peptide library for 30 minutes to one hour at 22°C
10 to allow complex formation between Flk-1 and peptide species within the library. The library is then washed to remove any unbound Flk-1 protein. If Flk-1 has been conjugated to alkaline phosphatase or horseradish peroxidase the whole library is poured into a petri dish
15 containing a substrates for either alkaline phosphatase or peroxidase, for example, 5-bromo-4-chloro-3-indoyl phosphate (BCIP) or 3,3',4,4"-diamnobenzidine (DAB), respectively. After incubating for several minutes, the peptide/solid phase-Flk-1 complex changes color, and can
20 be easily identified and isolated physically under a dissecting microscope with a micromanipulator. If a fluorescent tagged Flk-1 molecule has been used, complexes may be isolated by fluorescent activated sorting. If a chimeric Flk-1 protein expressing a
25 heterologous epitope has been used, detection of the peptide/Flk-1 complex may be accomplished by using a labeled epitope specific antibody. Once isolated, the identity of the peptide attached to the solid phase support may be determined by peptide sequencing.

30 In addition to using soluble Flk-1 molecules, in another embodiment, it is possible to detect peptides that bind to cell surface receptors using intact cells. The use of intact cells is preferred for use with receptors that are multi-subunits or labile or with
35 receptors that require the lipid domain of the cell

membrane to be functional. Methods for generating cell lines expressing Flk-1 are described in Sections 5.2.1. and 5.2.2. The cells used in this technique may be either live or fixed cells. The cells will be incubated
5 with the random peptide library and will bind to certain peptides in the library to form a "rosette" between the target cells and the relevant solid phase support/peptide. The rosette can thereafter be isolated by differential centrifugation or removed physically
10 under a dissecting microscope.

As an alternative to whole cell assays for membrane bound receptors or receptors that require the lipid domain of the cell membrane to be functional, the receptor molecules can be reconstituted into liposomes
15 where label or "tag" can be attached.

5.3.2. ANTIBODY PRODUCTION AND SCREENING

Various procedures known in the art may be used for the production of antibodies to epitopes of the
20 recombinantly produced Flk-1 receptor. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library. Neutralizing antibodies i.e., those which compete for the VEGF binding
25 site of the receptor are especially preferred for diagnostics and therapeutics.

Monoclonal antibodies that bind Flk-1 may be radioactively labeled allowing one to follow their location and distribution in the body after injection.
30 Radioactivity tagged antibodies may be used as a non-invasive diagnostic tool for imaging *de novo* vascularization associated with a number of diseases including rheumatoid arthritis, macular degeneration, and formation of tumors and metastases.
35

Immunotoxins may also be designed which target cytotoxic agents to specific sites in the body. For example, high affinity Flk-1 specific monoclonal antibodies may be covalently complexed to bacterial or
5 plant toxins, such as diphtheria toxin, abrin or ricin. A general method of preparation of antibody/hybrid molecules may involve use of thiol-crosslinking reagents such as SPDP, which attack the primary amino groups on the antibody and by disulfide exchange, attach the toxin
10 to the antibody. The hybrid antibodies may be used to specifically eliminate Flk-1 expressing endothelial cells.

For the production of antibodies, various host animals may be immunized by injection with the Flk-1
15 protein including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide,
20 surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

25 Monoclonal antibodies to Flk-1 may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein,
30 (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci., 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).
35 In addition, techniques developed for

the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse
5 antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be
10 adapted to produce Flk-1-specific single chain antibodies.

Antibody fragments which contain specific binding sites of Flk-1 may be generated by known techniques. For example, such fragments include but are not limited to:
15 the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science,
20 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to Flk-1.

5.4. USES OF Flk-1 CODING SEQUENCE

25 The Flk-1 coding sequence may be used for diagnostic purposes for detection of Flk-1 expression. Included in the scope of the invention are oligoribonucleotide sequences, that include antisense RNA and DNA molecules and ribozymes that function to inhibit translation of
30 Flk-1. In addition, mutated forms of Flk-1, having a dominant negative effect, may be expressed in targeted cell populations to inhibit the activity of endogenously expressed wild-type Flk-1.

35

5.4.1. USE OF Flk-1 CODING SEQUENCE IN DIAGNOSTICS AND THERAPEUTICS

The Flk-1 DNA may have a number of uses for the diagnosis of diseases resulting from aberrant expression of Flk-1. For example, the Flk-1 DNA sequence may be used in hybridization assays of biopsies or autopsies to diagnose abnormalities of Flk-1 expression; e.g., Southern or Northern analysis, including in situ hybridization assays.

10 The Flk-1 cDNA may be used as a probe to detect the expression of the Flk-1 mRNA. In a specific example described herein, the expression of Flk-1 mRNA in mouse embryos of different developmental stages was analyzed. Northern blot analysis indicated abundant expression of a
15 major 5.5 kb mRNA between day 9.5 and day 18.5, with apparent decline towards the end of gestation (FIG. 2A). In post-natal day 4-8 brain capillaries Flk-1 mRNA was found to be highly enriched compared to total brain RNA (FIG.2B), suggesting a role for Flk-1 in endothelial cell
20 proliferation.

To obtain more detailed information about the expression of Flk-1 during embryonic development and during the early stages of vascular development in situ hybridization experiments were performed as described in
25 Section 6.1.4. In situ hybridizations demonstrated that Flk-1 expression *in vivo* during embryonic mouse development is largely restricted to endothelial cells and their precursors (FIG. 3 and FIG. 4). Flk-1 is expressed in endothelial cells during physiological
30 processes that are characterized by endothelial cell proliferation and the temporal and spatial expression pattern found in the embryonic brain correlate precisely with the development of the neural vascular system as described by Bar (1980). Vascular sprouts originating in
35 the perineural plexus grow radially into the

neuroectoderm and branch there and these sprouts were found to express high amounts of Flk-1 mRNA (FIG. 5). In the early postnatal stages endothelial cell proliferation is still evident and Flk-1 is expressed, whereas in the
5 adult organism, after completion of the vascularization process, the decline in endothelial cell proliferation parallels a decrease in Flk-1 expression.

Also within the scope of the invention are oligo-ribonucleotide sequences, that include anti-sense RNA and
10 DNA molecules and ribozymes that function to inhibit the translation of Flk-1 mRNA. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA,
15 oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the Flk-1 nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism
20 of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and
25 efficiently catalyze endonucleolytic cleavage of Flk-1 RNA sequences.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which
30 include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure
35 that may render the oligonucleotide sequence unsuitable.

The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

- 5 Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as
- 10 for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which
- 15 incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.
- 20 Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends
- 25 of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

5.4.2. USE OF DOMINANT NEGATIVE Flk-1 MUTANTS IN GENE THERAPY

- 30 Receptor dimerization induced by ligands, is thought to provide an allosteric regulatory signal that functions to couple ligand binding to stimulation of kinase activity. Defective receptors can function as dominant
- 35 negative mutations by suppressing the activation and

response of normal receptors by formation of unproductive heterodimers. Therefore, defective receptors can be engineered into recombinant viral vectors and used in gene therapy in individuals that inappropriately express
5 Flk-1.

In an embodiment of the invention, mutant forms of the Flk-1 molecule having a dominant negative effect may be identified by expression in selected cells. Deletion or missense mutants of Flk-1 that retain the ability to
10 form dimers with wild type Flk-1 protein but cannot function in signal transduction may be used to inhibit the biological activity of the endogenous wild type Flk-1. For example, the cytoplasmic kinase domain of Flk-1 may be deleted resulting in a truncated Flk-1 molecule
15 that is still able to undergo dimerization with endogenous wild type receptors but unable to transduce a signal.

Abnormal proliferation of blood vessels is an important component of a variety of pathogenic disorders
20 such as rheumatoid arthritis, retinopathies and psoriasis. Uncontrolled angiogenesis is also an important factor in the growth and metastases of solid tumors. Recombinant viruses may be engineered to express dominant negative forms of Flk-1 which may be used to
25 inhibit the activity of the wild type endogenous Flk-1. These viruses may be used therapeutically for treatment of diseases resulting from aberrant expression or activity of Flk-1.

Expression vectors derived from viruses such as
30 retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of recombinant Flk-1 into the targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant
35 viral vectors containing Flk-1 coding sequence. See, for

example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing

- 5 Associates and Wiley Interscience, N.Y. Alternatively, recombinant Flk-1 molecules can be reconstituted into liposomes for delivery to target cells.

5.5. USE OF Flk-1 RECEPTOR OR LIGANDS

- 10 Receptor/ligand interaction between Flk-1 and VEGF is believed to play an important role in the signalling system during vascularization and angiogenesis. Abnormal proliferation of blood vessels is an important component of a number of diseases.

- 15 Expression of Flk-1 RNA correlates with the development of the brain and with endothelial cell proliferation suggesting that Flk-1 might be a receptor involved in mediation of signaling events in the vascularization process. VEGF has been shown to be a
20 mitogenic growth factor known to act exclusively on endothelial cell (Ferrara, N. and Henzel, W.J., 1989, Biochem. Biophys. Res. Comm. 161:851-858). Cross-linking and ligand binding experiments were performed, as described in Section 6.1.9 and 6.1.10 respectively, to
25 determine whether VEGF is a ligand for Flk-1 and the results indicate that Flk-1 is an authentic high affinity VEGF receptor (FIG 9).

- In one embodiment of the invention, ligands for Flk-1, the Flk-1 receptor itself, or a fragment
30 containing its VEGF binding site, could be administered in vivo to modulate angiogenesis and/or vasculogenesis. For example, administration of the Flk-1 receptor or a fragment containing the VEGF binding site, could competitively bind to VEGF and inhibit its interaction
35 with the native Flk-1 receptor in vivo to inhibit

angiogenesis and/or vasculogenesis. Alternatively, ligands for Flk-1, including anti-Flk-1 antibodies or fragments thereof, may be used to modulate angiogenesis and/or vasculogenesis. Agonists of VEGF activity may be used to promote wound healing whereas antagonists of VEGF activity may be used to inhibit tumor growth.

Depending on the specific conditions being treated, these agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition. Suitable routes may include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

6. EXAMPLE: CLONING AND EXPRESSION PATTERNS
OF Flk-1, A HIGH AFFINITY
RECEPTOR FOR VEGF

The subsection below describes the cloning and characterization of the Flk-1 cDNA clone. Northern blot and *in situ* hybridization analyses indicate that Flk-1 is expressed in endothelial cells. Cross-linking and ligand binding experiments further indicate that Flk-1 is a high affinity receptor for VEGF.

6.1. MATERIALS AND METHODS

6.1.1. cDNA CLONING OF Flk-1

DNA extracted from λ gt10 cDNA library of day 8.5 mouse embryos (Fahrner et al., 1987, EMBO. J. 6:1497-1508) was used as template for polymerase chain reaction (PCR; Saiki, R.K. et al., 1985 Science 230:1350-1354). In an independent approach cDNA of capillary endothelial cells that had been isolated from the brain of postnatal day 4-8 mice was used for amplification (Risau, W., 1990 In: development of the Vascular System. Issues Biomed. Basel Karger 58-68 and Schnürch et al., unpublished) Degenerated primers were designed on the basis of high amino acid homologies within the kinase domain shared by all RTKs (Wilks, A.F., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:1603-1607).

Full length cDNA clones of Flk-1 were isolated from another day 8.5 mouse embryo cDNA library, which had been prepared according to the method of Okayama and Berg (1983), and a day 11.5 mouse embryo λ gt11 library (Clonetech) using the ^{32}P -labeled (Feinberg, A.P. and Vogelstein, B. 1983 Anal. Biochem. 132:6-13) 210-bp PCR fragment.

6.1.2. MOUSE EMBRYOS

Balb/c mice were mated overnight and the morning of vaginal plug detection was defined as 1/2 day of gestation. For Northern blot analysis the frozen embryos were homogenized in 5 M guanidinium thiocyanate and RNA was isolated as described (Ullrich, A. et al., 1985, Nature 313:756-761). For *in situ* hybridization, the embryos were embedded in Tissue-Tek (Miles), frozen on the surface of liquid nitrogen and stored at -70C prior to use.

6.1.3. PREPARATION OF PROBES

The 5'-located 2619 bp of the receptor cDNA were subcloned in the pGem3Z vector (Promega) as an EcoR1/BamH1 fragment. The probe for Northern blot hybridization was prepared by labelling the cDNA fragment with α -³²PdATP (Amersham) by random hexanucleotide priming (Boehringer; Feinberg, A.P. and Vogelstein, B., 1983 Anal. Biochem. 132:6-13).

For *in situ* hybridization a single-strand antisense DNA probe was prepared as described by Schnürch and Risau (). The plasmid was linearized at the 3' end of the cDNA and a sense transcript was synthesized using SP6 RNA polymerase (Boehringer). The DNA was degraded using DNAase (RNAase free preparation, Boehringer Mannheim). With the transcript, a random-primed cDNA synthesis with a α -³⁵S dATP (Amersham) was performed by reverse transcription with MMLV reverse transcriptase (BRL). To obtain small cDNA fragments of about 100 bp in average suitable for *in situ* hybridization, a high excess of primer was used. Subsequently the RNA transcript was partially hydrolyzed in 100 mM NaOH for 20 minutes at 70°C, and the probe was neutralized with the same amount of HCl and purified with a Sephadex C50 column. After ethanol precipitation the probe was dissolved at a final specific activity of 5x10⁵ cpm. For control hybridization a sense probe was prepared with the same method.

6.1.4. RNA EXTRACTION AND NORTHERN ANALYSIS

Total cytoplasmic RNA was isolated according to the acidic phenol-method of Chromczynski and Sacchi (1987). Poly(A⁺) RNA aliquots were electrophoresed in 1.2% agarose formaldehyde (Sambrook, J. et al., 1989 Molecular Cloning: A Laboratory Manual 2nd ed. Cold Spring Harbor Laboratory Press) gels and transferred to nitrocellulose membranes (Schleicher & Schuell), Hybridizations were

performed overnight in 50% formamide, 5 x SSC (750mM sodium chloride, 75mM sodium citrate), 5 x Denhardt's (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% BSA) and -0.5% SDS at 42°C with $1-3 \times 10^6$ cpm-ml⁻¹ of ³²P-Random primed DNA probe, followed by high stringency washes in 0.2 x SSC, 0.5% SDS at 52°C. The filters were exposed for 4 to 8 days.

6.1.5. IN SITU HYBRIDIZATION

Subcloning postfixation and hybridization was essentially performed according to Hogan et al. (1986). 10 µm thick sections were cut at -18°C on a Leitz cryostat. For prehybridization treatment no incubation with 0.2M HCl for removing the basic proteins was performed. Sections were incubated with the ³⁵S-cDNA probe (5×10^4 cpm/µl) at 52°C in a buffer containing 50% formamide, 300 mM NaCl, 10 mM Tris-HCl, 10 mM NaPO₄ (pH 6.8), 5 mM EDTA, 0.02% Ficoll 400, 0.01% polyvinylpyrrolidone 0.02% BSA 10 mg/ml yeast RNA, 10% dextran sulfate, and 10 mM NaCl, 10 mM Tris-HCl, 10 mM NaPO₄ (pH 6.8), 5 mM EDTA, 10 mM DTT at 52°C). For autoradiography, slides were coated with Kodak NTB2 film emulsion and exposed for eight days. After developing, the sections were counterstained and toluidine blue or May-Grünwald.

6.1.6. PREPARATION OF ANTISERA

The 3' primed EcoRV/HindIII fragment comprising the 128 C-terminal amino acids of Flk-1 was subcloned in the fusion protein expression vector pGEX3X (Smith, D.B. and Johnson, K.S., 1990 Gene. 67:31-40; Pharmacia). The fusion protein was purified as described and used for immunizing rabbits. After the second boost the rabbits were bled and the antiserum was used for immunoprecipitation.

6.1.7. TRANSIENT EXPRESSION
OF Flk-1 IN COS-1 CELLS

Transfection of COS-1 cells was performed essentially as described by Chen and Okayama (1987 Mol. Cell. Biol. 7:2745-2752) and Gorman et al. (1989 Virology 171:377-385). Briefly, cells were seeded to a density of 1.0×10^6 per 10-cm dish and incubated overnight in DMEM containing 10% fetal calf serum (Gibco). 20 μ g of receptor cDNA cloned into a cytomegalovirus promotor driven expression vector was mixed in 0.5 ml of 0.25 M CaCl_2 , 0.5 ml of 2 x BBS (280 mM NaCl, 1.5 mM Na_2HPO_4 , 50 mM BES, pH 6.96 and incubated for 30 min at room temperature. The calcium phosphate/DNA solution was then added to the cells, swirled gently, and incubated for 18 hours at 37°C under 3% CO_2 . For ligand binding experiments, the cells were removed from the plate and treated as described below.

To obtain VEGF conditioned media, cells were transfected in 15-cm dishes. Media was collected after 48 h and VEGF was partially purified by affinity chromatograph using heparin High Trap TM columns (Pharmacia) and concentrated by ultrafiltration (Ferrara, N. and Henzel, W.J. 1989 Biochem. Biophys. Res. Comm. 161:851-858). The concentration of VEGF was determined by a ligand competition assay with bovine aortic endothelial cells.

For autophosphorylation assays, cells were seeded in 6-well dishes (2×10^5 cells per well), transfected as described above, and starved for 24 h in DMEM containing 0.5% fetal calf serum. The cells were then treated with 500 pM VEGF for 10 min. at 37°C or left untreated and were subsequently lysed as described by Kris et al. (1985). Flk-1 was immunoprecipitated with an antiserum raised in rabbits against the C-terminus of the receptor. The immunoprecipitates were separated on a 7.5% SDS

polyacrylamide gel, transferred to nitrocellulose, and incubated with a mouse monoclonal antibody directed against phosphotyrosine (5E2; Fendly, B.M. et al., 1990 Cancer Research 50:1550-1558). Protein bands were
5 visualized using horseradish peroxidase coupled goat anti-mouse antibody and the ECL™ (Amersham) detection system.

6.1.8. RADIOIODINATION OF VEGF

10 Recombinant human VEGF (5 µg; generously provided by Dr. H. Weich) was dissolved in 110 µl sodium phosphate buffer pH 7.6, and iodinated by the procedure of Hunter and Greenwood (1962). The reaction products were
15 separated from the labeled protein by passage over a sephadex G50 column, pre-equilibrated with phosphate buffered saline (PBS) containing 0.7% bovine serum albumin (BSA), and aliquots of the collected fractions were counted before and after precipitation with 20% trichloroacetic acid. The purity of the iodinated product
20 was estimated to be superior to 90%, as determined by gel electrophoresis, and the specific activity was 77000 cpm/ng. The bioactivity of the iodinated VEGF was confirmed by comparison with the bioactivities of native VEGF using the tissue factor introduction assay described
25 by Clauss, M. et al. (1990 J. Exp. Med. 172:1535-1545).

6.1.9. CROSSLINKING OF VEGF TO Flk-1

COS-1 cells transiently expressing Flk-1 and untransfected COS-1 cells were incubated with 200 pM ¹²⁵I-
30 VEGF at 4°C overnight, then washed twice with PBS and exposed to 0.5 mM disuccinimidyl suberate (DSS) in PBS for 1 h at 4°C. The cells were lysed, Flk-1 immunoprecipitated, and analyzed by electrophoresis on a 7% polyacrylamide gel followed by autoradiography.

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6.1.10. VEGF BINDING

Ligand binding experiments were performed as described previously (Schumacher, R. et al., 1991, J. Biol. Chem. 266:19288-19295), COS-1 cells were grown in a 15-cm culture dish in DMEM for 48h after transfection. Cells were then washed carefully with PBS and incubated with 5 ml of 25 mM EDTA in PBS for 10 min. Cells were then removed from the plate, washed once with binding buffer (DMEM, 25 mM HEPES, pH 7.5, 0.15% gelatin) and resuspended in 5 ml of binding buffer to determine the cell number. In a total volume of 500 μ l this cell suspension was incubated for 90 min at 15°C with 10 pM 125 I-VEGF, and increasing concentration of unlabeled ligand (from 0 to 7×10^{-9}), which was partially purified from conditioned media of COS-1 cells transiently expressing VEGF (164 amino acid form; Breier et al., 1992). After incubation, cells were washed with PBS 0.1% PBS in the cold. Free ligand was removed by repeated centrifugation and resuspension in binding buffer. Finally, the 125 I radioactivity bound to the cells were determined in a gamma counter (Riastar). Data obtained were analyzed by the method of Munson, P.J. and Rodbard, D. (1980 Anal. Biochem. 107:220-235).

25 6.2. RESULTS

6.2.1. ISOLATION OF Flk-1

To identify RTKs that are expressed during mouse development, PCR assays using two degenerate oligonucleotide primer pools that were designed on the basis of highly conserved sequences within the kinase domain of RTKs were performed (Hanks, S.K. et al. 1988, Science 241:42-52). DNA extracted from a λ gt10 cDNA library of day 8.5 mouse embryos (Fahrner, K. et al., 1987, EMBO. J., 6:1497-1508), a stage in mouse development at which many differentiation processes begin

was used as the template in the PCR assays. In a parallel approach, with the intention of identifying RTKs that regulate angiogenesis, similar primers were used for the amplification of RTK cDNA sequences from capillary
5 endothelial cells that had been isolated from the brains of postnatal day 4-8 mice, a time at which brain endothelial cell proliferation is maximal (Robertson, P.L. et al., 1985, Devel. Brain Res. 23:219-223). Both approaches yielded cDNA sequences encoding the recently
10 described fetal liver RTK, Flk-1 (Matthews, W. et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88:9026-9030). Based on amino acid homology, this receptor is a member of the type III subclass of RTKs (Ullrich, A. and Schlessinger, J. 1990, Cell 61:203-212) and is closely related to human
15 *flt*, which also contains seven immunoglobulin-like repeats in its extracellular domain in contrast to other RTKs of that subfamily, which contain only five such repeat structures (Matthews, W. et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88:9026-9030). Sequence comparisons of Flk-1
20 with KDR (Terman, B.I. et al., 1991, Oncogene 6:1677-1683) and TKr-C (Sarzani, R. et al., 1992, Biochem. Biophys. Res. Comm. 186:706-714) suggest that these are the human and rat homologous of Flk-1, respectively (Figure 1).

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6.2.2 EXPRESSION OF Flk-1 mRNA DURING EMBRYONIC DEVELOPMENT

As a first step towards the elucidation of the biological function of Flk-1, the expression of Flk-1
30 mRNA was analyzed in mouse embryos at different development stages. Northern blot hybridization experiments indicated abundant expression of a major 5.5 kb mRNA between day 9.5 and day 18.5, with an apparent decline towards the end of gestation (Figure 2A). In
35 postnatal day 4-8 brain capillaries Flk-1 mRNA was found

to be highly enriched compared to total brain mRNA (Figure 2B).

In situ hybridization experiments were performed to obtain more detail information about the expression of Flk-1 during different embryonal stages. A single-stranded antisense, 2619-nucleotide-long DNA probe comprising the Flk-1 extracellular domain was used as a probe because it generated the most specific hybridization signals. As an example, a parasagittal section of a day 14.5 embryo is shown in Figure 3. High levels of hybridization were detected in the ventricle of the heart, the lung, and the meninges; other tissues such as brain, liver, and mandible appeared to contain fewer cells expressing Flk-1 mRNA. Thin strands of Flk-1 expression were also observed in the intersegmental regions of the vertebrae and at the inner surface of the atrium and the aorta. Higher magnification revealed that the expression of Flk-1 seemed to be restricted to capillaries and blood vessels. Closer examination of the heart, for example, showed positive signals only in the ventricular capillaries and endothelial lining of the atrium (Figure 4A). In the lung, Flk-1 expression was detected in peribronchial capillaries, but was absent from bronchial epithelium (Figure 4D). The aorta showed strong hybridization in endothelial cells, but not in the muscular layer (Figure 4C).

6.2.3. EXPRESSION OF Flk-1 DURING ORGAN ANGIOGENESIS

The neuroectoderm in the telencephalon of a day 11.5 mouse embryos is largely avascular; the first vascular sprouts begin to radially invade the organ originating from the perineural vascular plexus (Bär, J., 1980, Adv. Anat. Embryol. Cell. Biol. 59:1-62; Risau, W. and Lemmon, V. 1988, Dev. Biol. 125:441-450). At this stage, expression of Flk-1 was high in the perineural vascular plexus

and in invading vascular sprouts, as shown in Figure 5A. These in situ hybridization analyses indicated that the proliferating endothelial cells of an angiogenic sprout expressed the Flk-1 mRNA. At day 14.5, when the
5 neuroectoderm is already highly vascularized, numerous radial vessels as well as branching vessels of the intraneural plexus contained large amounts of Flk-1 mRNA (Figure 5B). At postnatal day 4, when sprouting and endothelial cell proliferation is at its highest, strong
10 expression of Flk-1 mRNA was observed in endothelial cells (Figure 5C). Conversely, in the adult brain when angiogenesis has ceased. Flk-1 expression was very low (Figure 5D) and appeared to be restricted mainly to the choroid plexus (Figure 6). In the choroid plexus, cells
15 in the inner vascular layer expressed Flk-1 mRNA, while epithelial cells did not (Figure 6A, B).

The embryonic kidney is vascularized by an angiogenic process (Ekblom, P. et al., 1982, Cell Diff. 11:35-39). Glomerular and peritubular capillaries
20 develop synchronously with epithelial morphogenesis. In the postnatal day 4 kidney, in addition to other capillaries, prominent expression of Flk-1 was observed in the presumptive glomerular capillaries (Figure 7A). This expression persisted in the adult kidney (Figure 7C
25 and D) and then seemed to be more confined to the glomerular compared to the early postnatal kidney.

6.2.4. Flk-1 EXPRESSION IN ENDOTHELIAL CELL PROGENITORS

30 To investigate the possible involvement of Flk-1 in the early stages of vascular development, analysis of embryos at different stages during blood island formation were performed. In a sagittal section of the deciduum of a day 8.5 mouse embryo, Flk-1 expression was detected on
35 maternal blood vessels in the deciduum, in the yolk sac

and in the trophoctoderm. Flk-1 mRNA was also found in the allantois and inside the embryo, mainly located in that part where mesenchyma is found (Figure 8A). At a higher magnification of the maternal deciduum, high
5 levels of Flk-1 mRNA expression were found in the inner lining of blood vessels, which consist of endothelial cells (Figure 8B). In the yolk sac, hybridization signals were confined to the mesodermal layer, in which the hemangioblasts differentiate (Figure 8C). Figure 8D
10 shows a blood island at higher magnification, in which the peripheral angioblasts expressed a high level of Flk-1 mRNA.

6.2.5. Flk-1 IS A HIGH AFFINITY RECEPTOR FOR VEGF

15 Detailed examination of in situ hybridization results and comparison with those for VEGF recently reported by Breier, G. et al. (1992, Development 114:521-532) revealed a remarkable similarity in expression pattern. Furthermore, Flk-1 expression in the glomerular
20 endothelium and VEGF in the surrounding epithelial cells (Breier, G. et al., 1992, Development 114:521-532) raised the possibility of a paracrine relationship between these cells types and suggested therefore a ligand-receptor relationship for VEGF and Flk-1, respectively. In order
25 to test this hypothesis, the full-length Flk-1 cDNA was cloned into the mammalian expression vector pCMV, which contains transcriptional control elements of the human cytomegalovirus (Gorman, C.M. et al., 1989, Virology 171:377-385). For transient expression of the receptor,
30 the Flk-1 expressing plasmid was then transfected into COS-1 fibroblasts.

Specific binding of VEGF to the Flk-1 RTK was demonstrated by crosslinking and competition binding experiments. Purified ¹²⁵I-labeled VEGF was incubated with
35 COS-1 cells transfected with the pCMV-Flk-1 expression

vector. Crosslinking with DSS and subsequent analysis of immunoprecipitation, PAGE, and autoradiography revealed an approximately 220 kD band which was not detected in the control experiment with untransfected COS-1 cells and
5 is likely to represent the VEGF/Flk-1 receptor complex (Figure 9A). In addition, VEGF competed with ^{125}I -VEGF binding to Flk-1 expressing COS-1 cells (Figure 9B), whereas untransfected COS-1 cells did not bind ^{125}I -VEGF. The interaction of VEGF with the receptor on transfected
10 cells was specific, as PDGF-BB did not compete with binding of ^{125}I -VEGF. Analysis of the binding data revealed a Kd of about 10^{-10} M, suggesting that Flk-1 is a high affinity receptor of VEGF. This finding, together with the Flk-1 and VEGF in situ hybridization results
15 strongly suggests that Flk-1 is a physiologically relevant receptor for VEGF.

An autophosphorylation assay was performed to confirm the biological relevance of VEGF binding to the Flk-1 receptor. COS1 cells which transiently expressed
20 Flk-1 were starved in DMEM containing 0.5% fetal calf serum for 24h, stimulated with 0.5 mM VEGF, and lysed. The receptors were immunoprecipitated with the Flk-1 specific polyclonal antibody CT128, and the analyzed by SDS-PAGE and subsequent immunoblotting using the
25 antiphosphotyrosine antibody 5E2 (Fendly, B.M. et al., 1990, Cancer Research 50:1550-1558). As shown in Figure 10, VEGF stimulation of Flk-1 expressing cells led to a significant induction of tyrosine phosphorylation of the 180 kD Flk-1 receptor.

30 The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention, and any clones, DNA or amino acid sequences which are functionally equivalent are within the scope of the
35 invention. Indeed, various modifications of the

invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of
5 the appended claims.

It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for purposes of description.

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